

Compartmentation of glycolysis and glycogenolysis in the perfused rat heart

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ABSTRACT: Developing methods that can detect compartmentation of metabolic pathways in intact tissues may be important for understanding energy demand and supply. In this study, we investigated compartmentation of glycolysis and glycogenolysis in the isolated perfused rat heart using ¹³C NMR isotopomer analysis. Rat hearts previously depleted of myocardial glycogen were perfused with 5.5 mM [U-¹³C]glucose plus 50 mU/mL insulin until newly synthesized glycogen recovered to new steady-state levels (~60% of pre-depleted values). After a short wash-out period, the perfusate glucose was then switched to [1-¹³C]glucose, and glycolysis and glycogenolysis were stimulated by addition of glucagon (1 µg/ml). A ¹³C NMR multiplet analysis of the methyl resonance of lactate provided an estimate of pyruvate derived from glucose vs glycogen while a multiplet analysis of the C4 resonance of glutamate provided an estimate of acetyl-CoA derived from glycolytic pyruvate vs glycogenolytic pyruvate. These two indices were not equivalent and their difference was further magnified in the presence of insulin during the stimulation phase. These combined observations are consistent with functional compartmentation of glycolytic and glycogenolytic enzymes that allows pyruvate generated by these two processes to be distinguished at the level of lactate and acetyl-CoA. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: metabolic compartmentation; glycolysis; glycogenolysis; NMR isotopomer analysis

INTRODUCTION

Experimental evidence has been accumulating for functional organization of enzymes within the cytosol of cells.¹ One example of possible functional compartmentation came from the early work of Mowbray and

Ottaway,^{2–5} who first presented evidence for multiple, metabolic pools of pyruvate. Most early evidence for compartmentation came from differences in ¹⁴C specific activities of alanine vs lactate, metabolites that share pyruvate as a 3-carbon precursor.⁶ Because of differences in activity and regulation of alanine transaminase vs lactate dehydrogenase, it is not unusual to find differential alanine- and lactate-specific activities prior to metabolic steady state,⁷ but significant differences between the specific activities of these two metabolites at metabolic steady state has often been used as evidence for functional compartmentation of pyruvate.⁸ Peuhkurinen *et al.*⁹ proposed that one pool of pyruvate in the myocyte is associated more closely with glycolysis and tissue lactate, while a second ‘peripheral’ pool is in close communication with extracellular pyruvate and mitochondrial pyruvate. Tissue alanine is thought to be a better reflection of the second pyruvate pool than the first.⁶ This phenomenon was reevaluated in more recent ¹³C NMR studies of the heart.^{8,10–16} Hardin and Kushmerick¹⁵ demonstrated in smooth skeletal muscle that glycolytic intermediates derived from glucose do not fully mix with glycolytic intermediates derived from glycogen during simultaneous stimulation of both glycolysis and glycogenolysis. Furthermore, they observed that pyruvate derived from glycogen is oxidized in the citric

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Abbreviations used: C3S, singlet component of the methyl carbon resonance of lactate; C3D, doublet component of the methyl carbon resonance of lactate; C4S, singlet component of the carbon-4 resonance of glutamate; C4D34, doublet component of the carbon-4 resonance of glutamate representing spin-spin coupling between carbon-3 and carbon-4; C4D45, doublet component of the carbon-4 resonance of glutamate representing spin-spin coupling between carbon-4 and carbon-5; C4DQ, quartet component of the carbon-4 resonance of glutamate representing spin-spin coupling between carbon-4 and both carbon-3 and carbon-5; [1-¹³C]glucose, glucose enriched with ¹³C at the C1 carbon; gww, gram wet weight; HR, heart rate; KHB, Krebs–Henseleit bicarbonate; MSD rats, male Sprague–Dawley rats; PDH, pyruvate dehydrogenase complex; TCA cycle, tricarboxylic acid cycle; [U-¹³C]glucose, glucose enriched with ¹³C in all carbons.

acid cycle in preference to pyruvate derived from glucose. Zhao *et al.*,¹³ using ^1H NMR to monitor ^{13}C enrichment of alanine and lactate in isolated perfused rat hearts, observed differential labeling of these two metabolites (as reported by others), but also reported differences in NMR detectability of lactate and alanine that depended on perfusion conditions (glucose vs pyruvate perfused hearts). This differential NMR visibility suggested for the first time that compartmental pools of lactate and alanine in myocytes may exist in different physical microenvironments, clearly providing further evidence for functional organization of the cytosol. More recently, Chatham and Forder⁷ presented evidence for a non-exchanging pool of lactate and suggested that much of the earlier evidence for two separate pyruvate pools could be explained by two pools of lactate, one highly-sequestered, tightly-bound pool and another that is free and in rapid exchange with tissue pyruvate.

The idea of compartmentation of glycolysis and glycogenolysis in the heart is also evident in recent studies of regulation of carbohydrate metabolism in the perfused rat heart.^{17–22} Goodwin *et al.*^{18,23} reported preferential oxidation of glycogen vs exogenous glucose in isolated working rat heart and suggested that glycolysis from glycogen is better coupled to pyruvate oxidation. In the current study, we investigated further the extent to which glycolytic intermediates mix during simultaneous stimulation of glycolysis and glycogenolysis in isolated perfused rat hearts using different ^{13}C labeling patterns in glycogen and exogenous glucose. Our data demonstrate that the metabolic fate of pyruvate and the extent of mixing of intermediates along these parallel pathways are different in hearts stimulated by glucagon alone vs hearts stimulated by both glucagon and insulin.

MATERIALS AND METHODS

Materials

D_2O , 99.9%, $[1-^{13}\text{C}]$ - and $[\text{U}-^{13}\text{C}]$ glucose were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other chemicals were from Sigma (St Louis, MO, USA) and of the highest purity available.

Heart preparation

The study was performed under a protocol approved by the Animal Care and Use Committee at the University of Texas at Dallas. Male Sprague–Dawley rats weighing 200–220 g were injected with isoproterenol (5 mg/kg) subcutaneously 1 h before heart removal to deplete myocardial glycogen.^{13,24} Hearts were then rapidly excised and perfused using standard Langendorff techniques with a modified Krebs–Henseleit bicarbonate (KHB) buffer that contained (mM): NaCl (118), KCl (4.6), NaHCO_3

(25.3), CaCl_2 (1.2), MgSO_4 (1.16). The buffer was bubbled continuously with 95% O_2 –5% CO_2 .

Perfusion protocols

Hearts were attached to an all glass perfusion apparatus that fitted into the 51 mm bore of a standard vertical bore magnet and monitored by ^{13}C NMR. Initially the KHB perfusate was supplemented with 5.5 mM $[\text{U}-^{13}\text{C}]$ glucose plus 50 mU/ml insulin and perfusion was maintained until newly synthesized glycogen recovered to steady-state levels ($\sim 60\%$ of the pre-depleted value). After ~ 50 min, a comparison of the glycogen C1 and glucose C1 β resonances indicated that $[\text{U}-^{13}\text{C}]$ glycogen had risen to 18.1 ± 2.3 μmol glycogen/gww ($n = 4$). This value was in good agreement with glycogen determined enzymatically in freeze-clamped tissue, indicating that essentially all of the replenished glycogen was enriched with ^{13}C and that it was 100% NMR visible.²⁵ Hearts were then perfused for 5 min with KHB buffer lacking glucose and insulin to wash excess $[\text{U}-^{13}\text{C}]$ glucose from the NMR tube and perfusion lines. When the $[\text{U}-^{13}\text{C}]$ glucose resonances were no longer detectable by ^{13}C NMR, the perfusate was switched to one containing 5.5 mM $[1-^{13}\text{C}]$ glucose plus 2 mM dichloroacetic acid to fully stimulate PDH activity (group I hearts, $n = 6$) and 50 mU/ml of insulin (group II hearts, $n = 6$). After equilibration for an additional 10 min, 1 $\mu\text{g}/\text{ml}$ of glucagon was added to stimulate glycolysis and glycogenolysis.²⁶ Additional glucagon (0.1–0.5 $\mu\text{g}/\text{ml}$) was administered as necessary to maintain heart rate above baseline values (230 ± 11 bpm without insulin and 240 ± 11 bpm with insulin). The perfusions were terminated when the intensity of the $[\text{U}-^{13}\text{C}]$ glycogen ^{13}C NMR signal decreased by $\sim 50\%$. In all hearts this required a perfusion time on the order of 50 min, a period of time that is sufficient for the TCA cycle reactions to reach metabolic and isotopic steady state.²⁷ Thus, any labeling present in glutamate that originated during the initial perfusion with $[\text{U}-^{13}\text{C}]$ glucose (yielding C4D45 or C4Q multiplets) is completely depleted during subsequent perfusion with $[1-^{13}\text{C}]$ glucose.

Tissue extracts

At the conclusion of each NMR experiment, hearts were freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen. The frozen hearts were pulverized to a fine powder under liquid nitrogen using a pestle and mortar. This powder was homogenized with 3.6% cold perchloric acid and, after thawing, the homogenate was centrifuged at 15 000 rpm for 15 min at 5°C . The supernatant was neutralized ($\sim \text{pH}$ 7.4) using a minimum volume of KOH. This solution was centrifuged at 15 000 rpm for 20 min at 5°C and the resulting supernatant was freeze-dried, dissolved in 600 μl 99.9% D_2O , pH adjusted (pD ~ 7.0), filtered through a 5 mm filter, and the clear

solution was transferred into a 5 mm NMR tube for ^{13}C NMR analysis.

Tissue glycogen

Glycogen from freeze-clamped tissue was isolated using published procedures²⁸ and quantified using standard enzymatic methods.²⁹ Hearts from control animals ($n=3$) contained $30.1 \pm 3.2 \mu\text{mol}$ glycogen/gww while hearts from animals treated with isoproterenol 1 h prior to sacrifice ($n=3$) contained $3.5 \pm 0.3 \mu\text{mol}$ glycogen/gww. Perfusion of glycogen depleted hearts with 5.5 mM [^{13}C]glucose for approximately 1 h restored glycogen levels to $18.1 \pm 2.3 \mu\text{mol}$ glycogen/gww ($n=4$). All glycogen levels were in good agreement with values reported in previous studies.¹³

NMR spectroscopy

Broadband proton decoupled ^{13}C NMR spectra of intact hearts were collected on a 500 MHz Bruker GN spectrometer using a 18 mm $^{13}\text{C}/^1\text{H}$ probe. The hearts were positioned on the center of an 18 mm OD thin-walled NMR tube (Wilmad) and bathed in KHB perfusate. The temperature was maintained at 37°C by controlling both the perfusate temperature (using a water bath) and the air surrounding the 18 mm tube using a variable temperature accessory. ^{13}C NMR spectra were collected every 5 min using a 45° pulse and a 1 s delay between pulses. A total of 16 000 data points were collected to cover a sweep-

width of 28 000 Hz. Efficient broadband proton decoupling was achieved using WALTZ decoupling³⁰ at two power levels (low during the 1 s delay and high during acquisition). Shimming was performed on the ^{23}Na free induction decay (FID) after tuning the ^{13}C coil to ^{23}Na (132 MHz). Typical ^{23}Na line widths on the intact heart were 18–20 Hz. High-resolution ^{13}C NMR spectra of heart perchloric extracts were collected on the same spectrometer using a 5 mm broadband NMR probe. Each ^{13}C spectrum consisted of 65 000 data points covering a sweep-width of 28 000 Hz. Before Fourier transformation the FID's were zero-filled to 128 000 points and multiplied by a 0.5 Hz exponential.

Data analysis

Data are presented as mean \pm standard deviation (SD). ^{13}C NMR multiplet analysis was made using the deconvolution routine of the PC-based NMR program NUTSTM (Acorn NMR; Fremont, CA, USA). For each heart both the lactate C3 and the glutamate C4 resonances were fitted three times.

RESULTS

Perfusion of glycogen depleted hearts with 5.5 mM [^{13}C]glucose and 50 mU/ml insulin resulted in partial replenishment of myocardial glycogen ($\sim 60\%$ of pre-depleted levels). Figure 1 shows a stacked plot of ^{13}C NMR spectra collected every 5 min for a total duration of

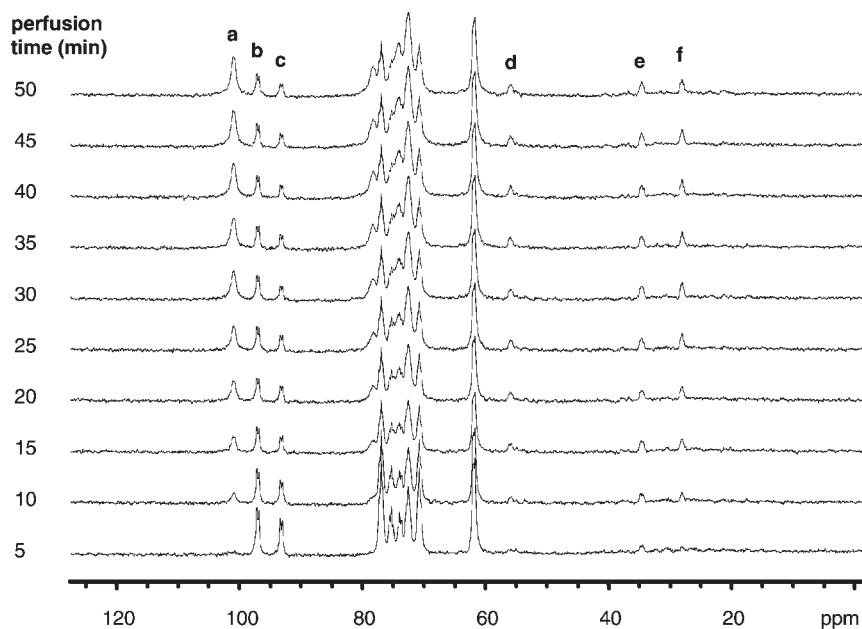


Figure 1. 125.7 MHz ^{13}C NMR spectra of an isolated perfused rat heart showing the production of [^{13}C]glycogen from [^{13}C]glucose over time. The resonances of interest are: glycogen C1 at 100.5 ppm (a); glucose C1 β (b) and C1 α (c) at 97.0 and 93.1 ppm, respectively; glutamate C2 at 55.5 ppm (d), C4 at 34.2 ppm (e) and C3 at 27.8 ppm (f). Other resonances include all other carbons from both [^{13}C]glucose and synthesized [^{13}C]glycogen

50 min. The C1 glycogen resonance (100.5 ppm) was clearly differentiated from the C1 β (97.0 ppm) and C1 α (93.1 ppm) resonances of [U- ^{13}C]glucose. The C2 (55.5 ppm), C4 (34.2 ppm) and C3 (27.8 ppm) resonances of glutamate were also visible, showing that some [U- ^{13}C]glucose produced [U- ^{13}C]pyruvate via glycolysis which then entered the citric acid cycle (TCA cycle). At the end of the 50 min perfusion period, the [U- ^{13}C]glycogen replenished hearts were perfused for 5 min with a glucose-free KHB buffer to eliminate all ^{13}C NMR signals arising from [U- ^{13}C]glucose. This was followed by switching to a KHB buffer containing 5.5 mM [1- ^{13}C]glucose and 2 mM dichloroacetate (to fully activate pyruvate dehydrogenase³¹). Hearts were equilibrated with this mixture for an additional 10 min before adding 1 $\mu\text{g}/\text{ml}$ of glucagon to stimulate both glycolysis and glycogenolysis.²⁶ Upon stimulation by glucagon, the average heart rate (HR) increased from 236 ± 11 to 268 ± 16 bpm and the ^{13}C signal of [U- ^{13}C]glycogen decreased progressively while both [3- ^{13}C]lactate (derived from glucose) and [U- ^{13}C]lactate (derived from glycogen) increased with time (not shown). Samples of perfusate (1–2 ml) were withdrawn during this period for later NMR analysis and the hearts were freeze-clamped after the glycogen signal intensity had decreased by $\sim 50\%$ (corresponding to approximately 50 min of perfusion). A sample ^{13}C NMR spectrum of a group I heart extract (no insulin) is shown in Fig. 2. The lactate methyl resonance centered at 20.7 ppm consisted of a singlet (S) reflecting lactate derived from exogenous [1- ^{13}C]glucose and a doublet (D) reflecting lactate derived from

[U- ^{13}C]glycogen. Note that the S component reflects only half of the lactate derived from [1- ^{13}C]glucose because a single molecule of [1- ^{13}C]glucose produces one [3- ^{13}C]lactate and one unenriched lactate. Thus, the lactate resonance area ratio, $2^*S/D$, directly reports the ratio of lactate produced from glucose vs lactate produced from glycogen during the stimulation phase. We refer to this ratio throughout the text as the 'lactate index'. Note that this index could potentially underestimate the contribution of glycogen to lactate formation owing to the $\sim 20\%$ of unenriched glycogen remaining in the tissue prior to replenishment of glycogen with [U- ^{13}C]glucose.

The glutamate C4 resonance centered at 34.2 ppm directly reports the distribution of ^{13}C in acetyl-CoA entering the TCA cycle. As shown in Fig. 2, the C4 resonance has four multiplet components, a singlet (S), two doublets with differing ^{13}C – ^{13}C couplings (D34 and D45), and a doublet-of-doublets or quartet (Q). As shown in numerous publications,^{32–34} C4S and C4D34 arise only from entry into the TCA cycle of [2- ^{13}C]acetyl-CoA, while C4D45 and C4Q arise only from entry of [1,2- ^{13}C]acetyl-CoA. Thus, the areas of these C4 multiplets report the relative amount of [3- ^{13}C]pyruvate (derived from [1- ^{13}C]glucose) vs [U- ^{13}C]pyruvate (derived from [U- ^{13}C]glycogen) that entered the TCA cycle during this same stimulation period. Hereafter, we refer to the glutamate C4 resonance area ratio, $2^*(S + D34)/(D45 + Q)$, as the 'glutamate index'. As before, factor 2 accounts for the fact that only half of the acetyl-CoA units derived from exogenous [1- ^{13}C]glucose are labeled

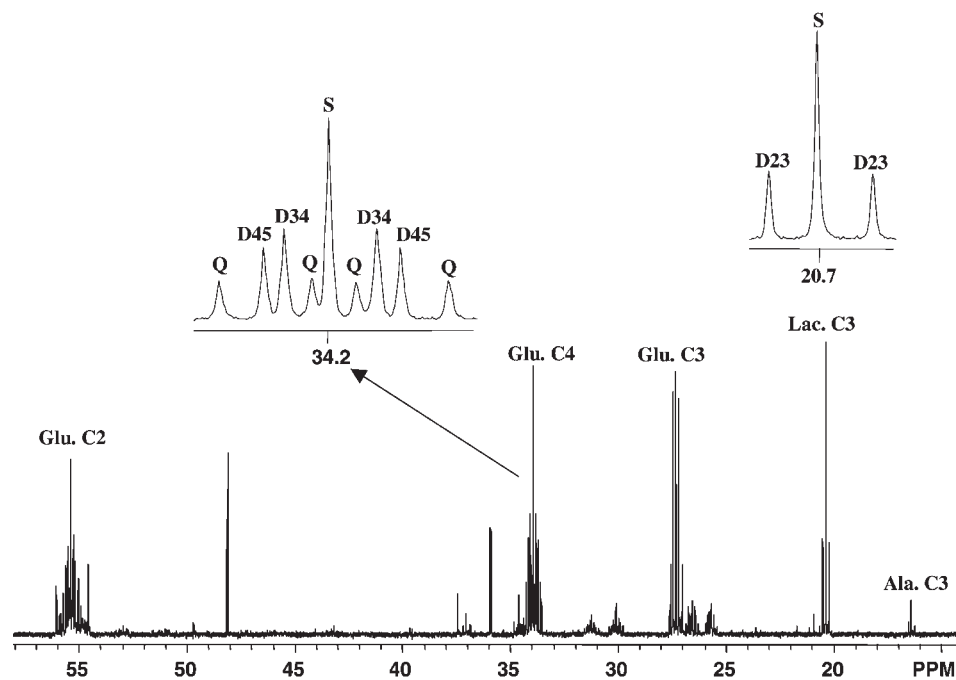


Figure 2. A 125.7 MHz ^{13}C NMR spectrum from the extract of a rat heart perfused with insulin present only during the glycogen synthesis. The insets show the expanded resonances of lactate C3 (20.7 ppm) and glutamate C4 (34.2 ppm). The glutamate index [$2^*(S + D34)/(D45 + Q)$] was significantly greater than the lactate index: [$2^*S/D$] ($p < 0.05$)

Table 1. Summary of heart rates and lactate and glutamate indices from group I hearts. Insulin was absent during the stimulation phase

Animal no.	Heart rate (bpm) baseline	Heart rate (bpm) stimulated	Lactate index (extracts)	Lactate index (perfusate)	Glutamate index (extracts)
1	230 ± 10	270 ± 20	3.12 ± 0.15	3.48 ± 0.17	3.72 ± 0.18
2	230 ± 10	270 ± 20	3.16 ± 0.15	3.50 ± 0.17	3.80 ± 0.19
3	240 ± 10	260 ± 10	3.52 ± 0.17	3.86 ± 0.19	4.34 ± 0.21
4	240 ± 10	260 ± 20	3.46 ± 0.17	3.80 ± 0.19	4.22 ± 0.21
5	250 ± 10	270 ± 20	3.38 ± 0.16	3.70 ± 0.18	4.10 ± 0.20
6	230 ± 20	280 ± 10	3.58 ± 0.17	3.82 ± 0.19	4.24 ± 0.21
Average	236 ± 11	268 ± 16	3.36 ^{a,b} ± 0.16	3.68 ^a ± 0.18	4.06 ^b ± 0.20

^a A paired *t*-test of the extract lactate index vs the perfusate lactate index ($t_{\text{stat}} = -18.53$, one-tail $t_{\text{critical}} = 2.57$ and $P(T \leq t) = 4.26 \times 10^{-6}$) showed significant differences ($p = 0.05$).

^b A paired *t*-test between lactate index in extracts and glutamate index in extracts ($p = 0.05$) with $t_{\text{stat}} = -10.37$, one-tail $t_{\text{critical}} = 2.57$ and $P(T \leq t) = 0.00014$ showed that glutamate index was significantly greater than lactate index.

Table 2. Summary of heart rates and lactate and glutamate indices in group II hearts. Insulin was present during the stimulation period

Animal no.	Heart rate (bpm) baseline	Heart rate (bpm) stimulated	Lactate index (extracts)	Lactate index (perfusate)	Glutamate index (extracts)
1	240 ± 10	320 ± 10	9.28 ± 0.46	9.88 ± 0.49	5.66 ± 0.28
2	230 ± 10	320 ± 10	9.90 ± 0.49	10.0 ± 0.50	5.92 ± 0.29
3	230 ± 10	310 ± 10	8.46 ± 0.42	8.90 ± 0.44	4.60 ± 0.23
4	250 ± 10	320 ± 30	9.82 ± 0.49	9.96 ± 0.49	5.82 ± 0.29
5	240 ± 10	340 ± 20	11.8 ± 0.59	12.1 ± 0.60	6.40 ± 0.32
6	250 ± 10	330 ± 20	8.48 ± 0.42	8.84 ± 0.44	4.40 ± 0.22
Average	240 ± 10	323 ± 20	9.60 ± 0.48 ^{a,b}	9.94 ± 0.49 ^a	5.46 ± 0.26 ^b

^a A paired *t*-test of two samples: lactate index in extracts and lactate index in perfusate with $t_{\text{stat}} = -4.39$, one tail $t_{\text{critical}} = 2.01$ and $P(T \leq t) = 0.00387$ showed that there was a significant statistical difference between lactate index in extracts and in perfusates and that lactate index in extracts was significantly lower than lactate index in the perfusate ($p = 0.05$).

^b A paired *t*-test between lactate index in extracts and glutamate index in extracts ($p = 0.05$) with $t_{\text{stat}} = 17.95$, one-tail $t_{\text{critical}} = 2.57$ and $P(T \leq t) = 4.94 \times 10^{-6}$ showed that the glutamate index was significantly lower than the lactate index ($p = 0.05$).

in carbon 2. As with the lactate index, the glutamate index could potentially underestimate the glycogen contribution owing to unlabeled glucose units remaining in the glycogen core.

A summary of ^{13}C NMR data from group I hearts is presented in Table 1. The lactate index reports that ~ 3.4 -fold more lactate was derived from $[1-^{13}\text{C}]\text{glucose}$ than from $[\text{U}-^{13}\text{C}]\text{glycogen}$ during the 50 min stimulation period. In comparison, the glutamate index reported that ~ 4.1 more acetyl-CoA was derived from $[3-^{13}\text{C}]\text{pyruvate}$ than from $[\text{U}-^{13}\text{C}]\text{pyruvate}$. The glutamate index was significantly higher ($p = 0.05$) than the lactate index (either tissue or perfusate lactate). Furthermore, the lactate index derived from spectra of lactate perfusate (3.68 ± 0.18) was significantly greater ($p = 0.05$) than lactate index measured in spectra of extracts (3.36 ± 0.16). This demonstrates that there is preferential export of lactate derived from $[1-^{13}\text{C}]\text{glucose}$ vs lactate derived from $[\text{U}-^{13}\text{C}]\text{glycogen}$ under these conditions.

The same protocol was repeated with insulin present (50 mU/ml) during the stimulation phase (group II). In this case, glucagon significantly increased both the heart rate and lactate production (Table 2). A ^{13}C NMR spectrum of a group II heart extract is shown in Fig. 3. The tissue lactate index reported by this spectrum in-

dicated that ~ 9.6 -fold more lactate was derived from exogenous $[1-^{13}\text{C}]\text{glucose}$ than from endogenous $[\text{U}-^{13}\text{C}]\text{glycogen}$. Also, a comparison of spectra from perfusate lactate vs extract lactate showed that group II hearts, like group I hearts, exported more lactate derived from $[1-^{13}\text{C}]\text{glucose}$ than lactate derived from $[\text{U}-^{13}\text{C}]\text{glycogen}$. Thus, even though lactate production from exogenous $[1-^{13}\text{C}]\text{glucose}$ was increased nearly 3-fold in the presence of insulin, there was a small but significant preference for export of glycolytic lactate.

An analysis of glutamate multiplets for group II hearts reported a glutamate index of 5.46 ± 0.26 , a value that was significantly higher than that measured in hearts stimulated by glucagon alone (group I hearts). This indicates that the pool of pyruvate entering the mitochondria was substantially different from the pool involved in formation of lactate. The observation that the glutamate index is smaller than either lactate index by nearly a factor of two (5.46 vs 9.60 or 9.94) shows that pyruvate derived from $[\text{U}-^{13}\text{C}]\text{glycogen}$ was, under these conditions, preferentially oxidized over pyruvate derived from $[1-^{13}\text{C}]\text{glucose}$. Also, the fact that the differential between the lactate and glutamate indices was even larger in this group of hearts shows that there is less mixing of pyruvate from the two glycolytic sources when insulin is present.

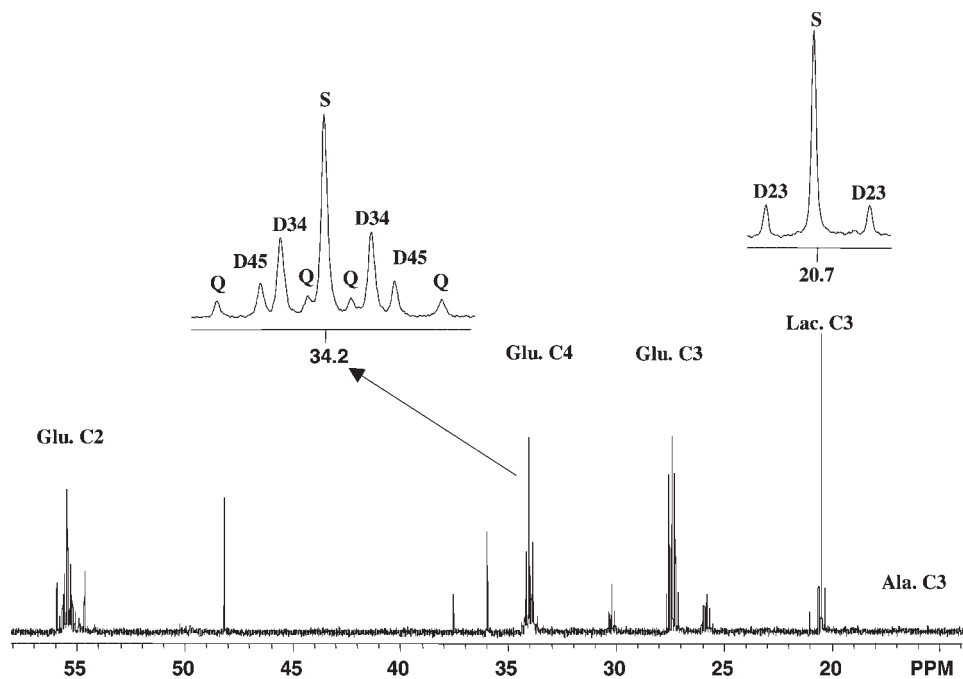


Figure 3. A 125.7 MHz ^{13}C NMR spectrum from the extract of a rat heart perfused with insulin present throughout the whole perfusion protocol. The insets show the expanded resonances of lactate C3 (20.7 ppm) and glutamate C4 (34.2 ppm). The glutamate index $[2 \cdot (S + D34) / D45 + Q]$ was significantly smaller than the lactate index: $[2 \cdot S / D]$ ($p < 0.05$)

DISCUSSION

This study tested the hypothesis that intermediates of the glycolytic and glycogenolytic pathways are not fully mixed in a perfused rat heart during hormonal stimulation of these pathways. Using a protocol similar to that used by Hardin and Kushmerick¹⁵ in a ^{13}C NMR study of hog carotid artery smooth muscle, myocardial glycogen was first depleted ($\sim 90\%$) then replenished with $[\text{U-}^{13}\text{C}]$ glucose to $\sim 60\%$ of pre-depleted levels prior to stimulation of both glycolysis and glycogenolysis using glucagon (group I) or glucagon plus insulin (group II). In group I hearts, the glutamate and lactate indices determined by ^{13}C NMR indicated that the pyruvate feeding the TCA cycle is not equivalent to the pyruvate feeding into lactate. As in the smooth muscle study,¹⁵ this provided evidence that glycolysis and glycogenolysis are functionally compartmentalized and pyruvate derived from these two processes does not completely mix in heart tissue. Incomplete mixing of two pyruvate pools supports the concept of two separate organized glycolytic pathways, one fed by glucose-6-phosphate (G6P) generated from endogenous glycogen and the other fed by G6P generated from exogenous glucose.³⁵ In group I hearts, pyruvate derived from exogenous glucose was oxidized in slight preference over pyruvate derived from endogenous glycogen. This result is opposite to that found in smooth muscle.¹⁵ Their conclusion was based upon the observation that insignificant $[\text{3-}^{13}\text{C}]$ lactate was detected in the ^{13}C NMR spectrum of hog carotid smooth muscle even

though glycogen preloaded with $[\text{1-}^{13}\text{C}]$ glucose was broken down during a prolonged contraction period. These authors presumed that absence of a $[\text{3-}^{13}\text{C}]$ lactate signal in the spectrum of the intact tissue was due to oxidation of the majority of $[\text{3-}^{13}\text{C}]$ pyruvate produced from glycogen in the citric acid cycle.

Recent reports have demonstrated that a non-exchanging pool of lactate can exist in the myocardium.^{7,8} In such studies, similarities were found between the ^{13}C -fractional enrichment of perfusate lactate and tissue alanine, as measured by ^1H NMR, and the ^{13}C -fractional enrichment of acetyl-CoA, as measured by the ^{13}C NMR spectrum of glutamate, but all three values were significantly higher than the ^{13}C -fractional enrichment of tissue lactate as measured by ^1H NMR. These differences were attributed to the presence of a 'metabolically inactive or less active' pool of tissue lactate that exchanges slowly with a 'metabolically active' pool of tissue lactate. Upon freeze-clamping the tissue, any sequestered pool of lactate would be released and hence detected in the ^1H NMR spectrum of tissue lactate. In our experiments, we compared the contribution of $[\text{1-}^{13}\text{C}]$ glucose vs $[\text{U-}^{13}\text{C}]$ glycogen to tissue and perfusate lactate by ^{13}C NMR so the spectrum in this case would be insensitive to any non-exchanging, metabolically inactive pool of tissue lactate. Nevertheless, our observation that the tissue and perfusate lactate indices differed in both experiments (stimulation by glucagon alone vs glucagon plus insulin) indicates that the myocardium can distinguish lactate derived from the two carbohydrate sources.

In group II hearts, insulin was present throughout the experiment. In this case, a greater difference between the lactate and glutamate indices was observed, indicating even less mixing of glycolytic and glycogenolytic intermediates had occurred. The contribution of glycolytic/glycogenolytic pyruvate to oxidative metabolism was greater in this experiment, but this was largely determined by the enhanced glycolytic flux (compared with glycogenolytic flux) induced by insulin. However, the fact that the cytosolic glycolytic/glycogenolytic pyruvate ratio (as reported by tissue lactate) was 9.60 ± 0.46 while the mitochondrial glycolytic/glycogenolytic pyruvate ratio (as reported by glutamate) was only 5.46 ± 0.26 indicates there was a rather dramatic switch in preference for oxidation of pyruvate derived from glycogen in the presence of insulin. We considered the possibility that the high lactate index was due to contribution from natural abundance ^{13}C signal, but the singlet in the lactate C2 was negligible, indicating an insignificant natural abundance contribution. These results therefore suggest a biological effect of insulin. Horowitz and Pearson³⁶ have shown that the activity of diffusive substrates in the cytosol of oocytes is altered by changes in either the molecular freedom or location of water and that this process is modulated by insulin. Such a mechanism could be responsible for the influence of insulin on the exchange or mixing of glycolytic and glycogenolytic intermediates we observed here.

Two alternative models might be used to describe simultaneous flow of glucose-6-phosphate (G6P) derived from glucose *versus* glycogen to pyruvate in the rat heart (Fig. 4). In model I, all G6P, regardless of origin, enters a single glycolytic pathway and this would result in a single pool of pyruvate. It is also assumed that all glycolytic

intermediates between G6P and pyruvate fully mix along this pathway. Note that this model does not require any particular organization of glycolytic enzymes as represented by the 'pipe column' of Fig. 4 (i.e. the enzymes could be randomly distributed throughout the cytosol). Nevertheless, the single pool of pyruvate generated from glucose and glycogen would either produce lactate or acetyl-CoA for subsequent oxidation in the TCA cycle. In this model, under steady-state metabolic conditions, the tissue lactate, perfusate lactate and glutamate indices would be equal.

In model II, the cytosol has two functionally organized glycolytic pathways, one of which converts G6P derived from glucose into pyruvate and another that converts G6P derived from glycogen into pyruvate. In this model, it is assumed that none of the glycolytic intermediates between G6P and pyruvate mix and that two separate pools of pyruvate are generated, perhaps destined for different metabolic fates. This would ultimately be reported by the spectra of lactate and glutamate. It is important to point out, however, that functional compartmentation of two separate, organized 'pipes' of glycolytic enzymes does not necessarily insure unequal lactate and glutamate indices. Thus, the unequal indices measured here using two different stimulation protocols (glucagon \pm insulin) does lend support to model II, but the fact that pyruvate derived from both glucose and glycogen does appear in tissue lactate, perfusate lactate, and tissue glutamate under all circumstances indicates that mixing of glycolytic intermediates does occur to some extent, at least at the level of pyruvate.

The increase in the lactate and glutamate indices observed for the glucagon + insulin perfused hearts could also be the result of an increase in glycogen turnover in

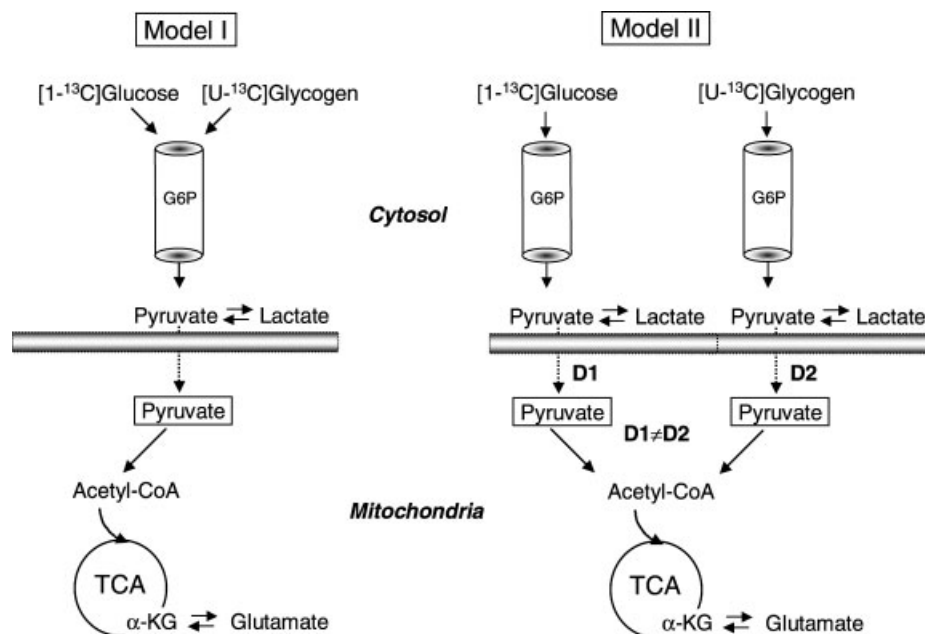


Figure 4. Schematic models for functional compartmentation of glycolytic and glycogenolytic intermediates in the perfused rat heart

the presence of insulin.²⁶ An increased glycogen turnover would promote the synthesis of [1-¹³C]glycogen from exogenous [1-¹³C]glucose, which could subsequently generate [3-¹³C]lactate via glycogenolysis and thereby increase both the lactate and glutamate indices. An increased glycogen turnover causes an overestimation of glycolytic flux since all [1-¹³C]glucosyl units in this experiment are assumed as being directly derived from exogenous [1-¹³C]glucose provided in the perfusate and not from newly synthesized [1-¹³C]glycogen. Goodwin *et al.*²⁶ found that only 7.5% of exogenous glucose taken into heart tissue was directed toward glycogen synthesis when perfused with 10 mU/mL insulin. They also report that the rate of glycogen synthesis is low compared with glycolytic flux. This suggests that any correction to the lactate and glutamate indices due to glycogen turnover would be small and insufficient to alter the conclusions described above.

The small differences between perfusate and tissue lactate indices could be attributed to a relatively small pool of lactate not in complete exchange with a major cytosolic pool. In recent work by Chatham *et al.*,⁸ evidence was presented for the existence of two separate pathways for lactate uptake and release in the perfused heart. Lactate generated via glycolysis reportedly accounts for most of the released lactate whereas exogenous lactate was preferentially oxidized. The existence of two separate intracellular pyruvate pools was also postulated for explaining the intermediary metabolism in pancreatic β -cells.³⁷ Here, one pyruvate pool is involved in carboxylation/decarboxylation exchanges with TCA intermediates (probably OAA and malate), while another functionally separate pyruvate pool provides acetyl-CoA for entry into the TCA cycle.

The idea of functional metabolic compartmentation is often invoked in the current literature, yet experimental tools capable of probing its existence in intact tissues are few. The results presented in this work using ¹³C tracers and ¹³C NMR are consistent with the existence of intracellular compartmentation of pyruvate³⁸ and/or other glycolytic intermediates but one cannot rule out the possibility that these observations are at least in part due to tissue heterogeneity.³⁹ The extent to which this contributes to the results reported here could be tested by performing future experiments on isolated myocytes.

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